Antimicrobial Activity of Fractions and Compounds from *Calophyllum brasiliense* (Clusiaceae/Guttiferae)

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Calophyllum brasiliense (Clusiaceae/Guttiferae) is a native Brazilian medicinal plant traditionally used against several diseases, including infectious pathologies. Crude methanolic extracts (CME) and two fractions, denoted non-polar (soluble in chloroform) and polar (non-soluble in chloroform), were prepared from different parts of the plant (roots, stems, leaves, flowers and fruits) and studied. The following compounds were isolated and tested against pathogenic bacteria and yeasts by determination of the minimal inhibitory concentration (MIC): brasiliensic acid (**1**), gallic acid (**2**), epicatechin (**3**), protocatechuic acid (**4**), friedelin (**5**) and 1,5-dihydroxyxanthone (**6**). The results indicated that all the parts of the plant exhibited antimicrobial activity against Gram-positive bacteria, which are selectively inhibited by components of *C. brasiliense.* No activity was observed against Gram-negative bacteria and yeasts tested. Regarding the isolated compounds, substance **4** showed antimicrobial activity against all the tested microorganisms, whereas compound **6** exhibited antimicrobial activity only against Gram-positive bacteria. The results from the current study confirm and justify the popular use of this plant to treat infectious processes.

Key words: Calophyllum brasiliense, Antimicrobial Activity, Phenolic Compounds

Introduction

The genus *Calophyllum* (Clusiaceae/Guttiferae) is composed of about 180–200 different species confined to the warm humid tropics of the world (Stevens, 1980). Extensive chemical investigation of this genus has resulted in the isolation of a wide variety of natural products, including xanthones, coumarins, biflavonoids, chalcones, benzofurans and triterpenes (Da Silva *et al.*, 2001; Ito *et al.*, 2002, 2003; Oger *et al.*, 2003; Isaias *et al.*, 2004).

Some of these species are frequently employed in folk medicine to treat several injuries (Sartori *et al.*, 1999). Several experimental studies have reported that extracts and/or the oil of several species of this genus are potential antibacterial (Bhat *et al.*, 1954; Potti and Kurup, 1970; Mahmud *et al.*, 1998; Ali *et al.*, 1999; Dharmaratne *et al.*, 1999; Khan *et al.*, 2002; Sakagami *et al.*, 2002) and antifungal sources (Morel *et al.*, 2002; Oger *et al.*, 2003)

In Latin America, one of the most abundant and widely distributed species is *Calophyllum brasiliense*, a large tree native to the tropical forest which thrives from Brazil to Mexico, known in Brazil as "Bari, Guarandi, Guanandi or Jacareuba" (Correa, 1978; Da Silva *et al.*, 2001; Mesía-Vela *et al.*, 2001). This plant has been used in folk medicine to treat bronchitis, gastric and hepatic disturbances (Sartori *et al.*, 1999), pain (Lewis, 1977), inflammation, diabetes, hypertension (Duke and Martinez, 1994), diarrhea, herpes (Rutter, 1990), rheumatism, varicose, hemorrhoids and chronic ulcer (Correa, 1978). Other studies have focused on a group of coumarins and xanthones, which act as potent inhibitors of HIV-1 reverse transcriptase (Dharmaratne *et al.*, 2002; Ito *et al.*, 2002). Recently several compounds (coumarins and xanthones) from this plant were examined for cancer chemopreventive activity (Ito *et al.*, 2002) and trypanomicidal activity (Abe *et al.*, 2004). However, to the best of our knowledge, no study has been carried out on the possible antimicrobial action of this plant.

In the present investigation, we examined the possible antimicrobial effects of extracts (methanolic), some fractions (non-polar, ethyl acetate, chloroform, polar) and some pure compounds such as brasiliensic acid (**1**), gallic acid (**2**), epica-

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techin (**3**), protocatechuic acid (**4**), friedelin (**5**), and 1,5-dihydroxyxanthone (**6**) obtained from different parts of *Calophyllum brasiliense*.

Material and Methods

Plant material

Calophyllum brasiliense was collected from the gardens of the Federal University of Santa Catarina (Florianópolis, SC, Brazil). Roots, flowers and fruits were collected in April, September and December 2001 whereas stems and leaves in December 1998. The material was classified by Dr. Ademir Reis (Department of Botany, UFSC). A voucher specimen was deposited in the Barbosa Rodrigues Herbarium (Itajaí, SC, Brazil) under number VC Filho 007.

Phytochemical analysis

Air-dried material from different parts of the plant (roots, stems, leaves, flowers and fruits) were powdered and macerated separately with methanol at room temperature for 7 d. After filtration, the solvent was removed by rotary evaporation under reduced pressure. The crude methanolic extracts (CME) were dissolved in chloroform. The soluble parts were denominated as non-polar and the non-soluble parts as polar fractions. They were preliminarily analysed by TLC and specific reagents, according to the methodology previously described (Cechinel Filho and Yunes, 1998).

The respective fractions (non-polar and polar) were chromatographed with a silica gel (Merck) column using an appropriate solvent system, successively to afford the pure compounds: brasiliensic acid (**1**), gallic acid (**2**), epicatechin (**3**), protocatechuic acid (**4**), friedelin (**5**), and 1,5-dihydroxyxanthone (**6**), as previously described (Da Silva *et al.*, 2001; Isaias *et al.*, 2004). The purity of all the isolated compounds was examined by TLC using Merck silica pre-coated aluminium plates of 200 μm thickness with several solvent systems of different polarities. Spots were visualized by short-wave UV ligth, sulfuric acid, sulfuric anisaldehyde and $FeCl_3$ reagents.

The identification of isolated compounds was performed by analyses of melting points, IR spectra, ^1H and ^{13}C NMR spectra as well as the comparison of physical data with those reported.

Microorganisms

For antimicrobial activity determinations the following microorganisms were used: *Bacillus cereus* (ATCC 14579), *Enterobacter cloacae* (ATCC 35030), *Escherichia coli* (ATCC 11775), *Proteus mirabilis* (ATCC 14273), *Pseudomonas aeruginosa* (ATCC 35032), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 6538P), *Staphylococcus saprophyticus* (ATCC 35552) and *Streptococcus agalactiae* (ATCC 13813), *Candida albicans* (ATCC 10231) and *Candida tropicalis* (ATCC 7349). These were purchased from the tropical culture collection of the "Fundação Tropical de Pesquisa e Tecnologia André Tosello" (The André Tosello Tropical Fondation for Research & Technology), Campinas, State of São Paulo, Brazil.

Quantitative antimicrobial evaluation

The susceptibility tests were performed using the agar dilution assay – a modified version of the National Committee for Clinical Laboratory Standards (1993).

The test components (extracts, fractions and compounds) were dissolved in dimethyl sulfoxide (DMSO) and added to twofold serial Mueller-Hinton agar medium (Merck) for the bacteria and Sabouraud dextrose agar medium (Merck) for the yeasts to achieve a final volume of 1 ml and concentrations ranging from 10 μg/ml to 1000 μg/ml. Drugs (amoxicillin and vancomycin) (Sigma) as well as growth and blank (media and solvent 2%) controls were added to each test. The microbial inoculum sizes were 1.5×10^6 cells/ml for bacteria (NCCLS, 1993) and 10^6 cells/ml for yeasts (Espinel-Ingroff and Pfaller, 1995).

The minimal inhibitory concentration (MIC) values were taken as the lowest concentration of extracts, fractions, or compounds that inhibited the growth of the organism after 24 h of incubation at 37 °C.

Results and Discussion

Considering the evolution of resistance genes to antibiotics of microbial origin and non-antibiotic chemicals (Köhler *et al.*, 1999; Lee *et al.*, 2003), plant materials have become the subject of public attention and therefore the pharmaceutical industry is moving away from drug discovery or screening towards compounds isolated by medicinal plants.

Medicinal plants are used in traditional medicine for several purposes. The secondary metabolites produced by plants constitute a source of bioactive substances and nowadays scientific interest has increased due to the search for new drugs of plant origin (Basile *et al.*, 2000; Paiva *et al.*, 2003).

The preliminary study carried out with *C. brasiliense* (data unpublished), showing its antimicrobial activity, encouraged us to continue the antimicrobial and chemical investigations in order to identify the active principles of this plant.

Thus, we initially prepared the crude methanolic extracts (CME) and two fractions, denoted non-polar (soluble in chloroform) and polar (non-soluble in chloroform) from different parts of the plant (roots, stems, leaves, flowers and fruits) in order to determine the location of possible active compounds. However, the flower was not studied in detail because of its low yield. The following compounds were isolated and tested: brasiliensic acid (**1**), gallic acid (**2**), epicatechin (**3**), protocatechuic acid (**4**), friedelin (**5**) and 1,5-dihydroxyxanthone (**6**) (Fig. 1).

The majority of the clinically useful antibiotics is active against the test strains at a level of at least 10 μg/ml. A pure agent which is not active at least at 100 μg/ml is unlikely to be a serious candidate for clinical use unless it is active against a recalcitrant organism or is comparatively nontoxic (Mitscher *et al.*, 1972). On this basis, tests on plant extracts were carried out at 1000 μg/ml and 100 μg/ml.

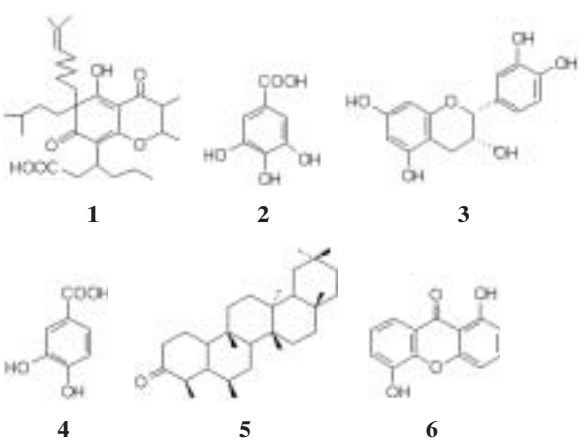

Fig. 1. Molecular structures of the compounds [brasiliensic acid (**1**); gallic acid (**2**); epicatechin (**3**); protocatechuic acid (**4**); friedelin (**5**); 1,5-dihydroxyxanthone (**6**)] isolated from *Calophyllum brasiliense.*

Table I. Antimicrobial activity of extract and fractions of *Calophyllum brasiliense* against Gram-positive bacteria, expressed as minimal inhibitory concentration (MIC).

Material tested	MIC [μg/ml]			
	B.c.	*S.a.*	*S.s.*	*S.ag.*
Roots CME	> 1000	300	900	> 1000
Roots Non-Polar	> 1000	300	> 1000	> 1000
Roots Polar	400	400	> 1000	> 1000
Stems CME	> 1000	> 1000	> 1000	> 1000
Stems Non-Polar	> 1000	300	> 1000	> 1000
Stems Polar	600	600	400	500
Leaves CME	900	300	500	700
Leaves Non-Polar	400	300	900	100
Leaves Polar	1000	600	700	700
Flowers CME	300	200	800	1000
Fruits CME	> 1000	> 1000	> 1000	> 1000
Fruits Non-Polar	> 1000	400	700	> 1000
Fruits Polar	> 1000	500	1000	700
Vancomycin	0.7	2	2	0.8

Crude methanolic extract (CME); fraction non-polar (Non-Polar); fraction polar (Polar); *Bacillus cereus (B.c.); Staphylococcus aureus (S.a.); Staphylococcus saprophyticus (S.s.); Streptococcus agalactiae (S.ag.).*

Extracts with MIC values less than 100 μg/ml were considered with good antimicrobial activity, whereas MIC values of 100 to 500 μg/ml were considered moderately active, 500 to 1000 μg/ml were considered weak active, and more than 1000 μg/ml were considered inactive.

As show in Table I, all parts of the plant (roots, stems, leaves and flowers) exhibited antimicrobial activity, the leaves, however, presented a larger prevalence of activity.

The results indicate that Gram-positive bacteria are selectively inhibited by components of *C. brasiliense.* The pattern of chemical selectivity towards Gram-positive bacteria is not restricted to compounds from plants, but is a general phenomenon observed among most antibiotics (Basile *et al.*, 2000; Schaechter *et al.*, 1999).

No activity was observed against Gram-negative bacteria (*Enterobacter cloacae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) and yeasts (*Candida albicans* and *Candida tropicalis*). This can be explained because the outer membrane of Gram-negative bacteria is known to present a barrier to the penetration of numerous antibiotic molecules, and the periplasmic space contains enzymes which are able of breaking down foreign molecules introduced from outside (Schaechter *et al.*, 1999; Duffy and Power, 2001; Sartori *et al.*, 2003).

Table II. Antimicrobial activity of compounds of *Calophyllum brasiliense* against bacteria and yeasts, expressed as minimal inhibitory concentration (MIC).

Compound	MIC [μg/ml]										
	Gram-positive bacteria				Gram-negative bacteria					Yeasts	
	B.c.	*S.a.*	*S.s.*	*S.ag.*	*E.cl.*	*E.c.*	*Ps.a.*	*P.m.*	*S.t.*	*C.a.*	*C.t.*
1	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
2	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
3	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
4	500	200	200	200	400	400	800	500	700	500	400
5	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
6	700	200	200	500	800	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Amoxicillin	nt	nt	nt	nt	nt	6	nt	nt	1	nt	nt
Vancomycin	0.7	2	2	0.8	nt	nt	nt	nt	nt	nt	nt

Brasiliensic acid (**1**) [100 μg/ml = 195 μM]; gallic acid (**2**) [100 μg/ml = 588 μM]; epicatechin (**3**) [100 μg/ml = 344 μM]; protocatechuic acid (**4**) [100 μg/ml = 649 μM]; friedelin (**5**) [100 μg/ml = 235 μM]; 1,5-dihydroxyxanthone (**6**) [100 μg/ml = 438 μM]; amoxicillin [100 μg/ml = 273 μM]; vancomycin [100 μg/ml = 67 μM]; not tested (nt); *Bacillus cereus (B.c.); Staphylococcus aureus (S.a.); Staphylococcus saprophyticus (S.s); Streptococcus agalactiae (S.ag.); Enterobacter cloacae (E.cl.); Escherichia coli (E.c.); Pseudomonas aeruginosa (Ps.a.); Proteus mirabilis (P.m.); Salmonella typhimurium (S.t.); Candida albicans (C.a.); Candida tropicalis (C.t.).*

Many Gram-negative organisms also exhibit intrinsic high-level resistance to a range of antimicrobial agents and support a role for the outer membrane and active efflux as a barrier to antibiotics (Nikaido, 1989; Köhler, 1999; Van Bambeke *et al.*, 2003).

Regarding the isolated compounds (Table II), protocatechuic acid (**4**) showed antimicrobial activity against all the tested microorganisms: Gram-positive, Gram-negative bacteria and yeasts.

1,5-Dihydroxyxanthone (**6**) exhibited some specific antimicrobial activity, being active only against Gram-positive bacteria.

Brasiliensic acid (**1**) was only tested up to a the maximum concentration of 100 μg/ml, in which no activity was detected. The other compounds (**2**, **3** and **5**) did not exhibit activity against the microorganisms tested until the concentration of 1000 μg/ml.

The activities detected in the extracts and fractions of the different parts of *C. brasiliense* suggest a synergistic action of some compounds, as well as the presence of other bioactive compounds that already had been isolated from the leaves of this plant as coumarins (Ito *et al.*, 2003), flavonoids (Sartori *et al.*, 1999; Da Silva *et al.*, 2001), xanthones (Reyes-Chilpa *et al.*, 1997; Sartori *et al.*, 1999; Ito *et al.*, 2002) etc., identified in other works, and recognized as antimicrobial agents against several microorganisms (Cowan, 1999).

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